Incorporation of Tryptophan Analogues into Staphylococcal Nuclease, Its V66W Mutant, and Δ137−149 Fragment: Spectroscopic Studies†

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ABSTRACT: We have biosynthetically incorporated several tryptophan analogues into three forms of Staphylococcal nuclease to investigate the spectroscopic characteristics of these "intrinsic" probes and their effect on the structure of the proteins. The set of tryptophan analogues includes 5-hydroxytryptophan, 7-azatryptophan, 4-fluorotryptophan, 5-fluorotryptophan, and 6-fluorotryptophan. 5-Hydroxytryptophan and 7-azatryptophan have red-shifted absorbance spectra, and the latter has a red-shifted fluorescence, which is very sensitive to its environment (being heavily quenched in water). The fluorotryptophans can serve as ^{19}F NMR probes, and 4-fluorotryptophan has a very low fluorescence quantum yield, thus making it a "knock-out" fluorescence analogue. The set of proteins studied includes wild-type nuclease, which has a single tryptophan site at position 140; its V66W mutant, which has a second tryptophan at position 66; and the Δ137−149 fragment, V66W′, which only has a tryptophan at position 66. The environments of positions 66 and 140 are significantly different; position 140 is near the end of the long C-terminal α-helix and is moderately solvent-exposed, whereas position 66 is in the β-barrel core region of the protein and is surrounded by apolar side chains. Absorbance and ^{19}F NMR spectra are used to estimate the extent of analogue incorporation for each protein. Steady-state and time-resolved fluorescence data are reported to characterize the emission of the analogues in these positions in the three proteins and to develop the use of the analogues as probes of protein structure and dynamics. Circular dichroism spectra are reported to show that, in all but a couple of cases, the secondary structure of the proteins containing the analogues is not significantly perturbed by the probes. Additionally, fluorescence anisotropy decay data show the variants of wild-type nuclease to have a rotational correlation time similar to that of tryptophan-containing nuclease.

There has been a recent flurry of studies in which amino acid analogues are biosynthetically incorporated into proteins to serve as probes for characterizing function and to determine the degree to which these analogues perturb the structure and stability of the proteins. The methods of incorporation include cell-free systems (*1*, *2*) and in vivo expression systems (*3*−*6*).

The amino acid tryptophan (Trp) is frequently targeted for substitution with an analogue. The reasons for this choice are that there are usually few tryptophans in a protein, there are several possible analogues of Trp, and some of these have different spectroscopic properties. What seems to be the earliest biosynthetic incorporation of a Trp analogue was the work of Pardee et al. (*7*), who demonstrated the incorporation of 7-azatryptophan and 2-azatryptophan into the total protein of a Trp auxotroph of *Escherichia coli*. The first report of the purification of an active protein containing a Trp analogue was by Schlessinger (*8*), who biosynthetically incorporated 7-azatryptophan and 2-azatryptophan into alkaline phosphatase. The laboratories of Sykes and Ho then developed the strategy of incorporating fluorotryptophan into proteins, for use as a ^{19}F NMR probe (*9*, *10*). In recent years, groups led by Szabo (*3*, *4*) and Ross (*5*, *6*, *11*) have further extended this area by incorporating 5-hydroxytryptophan and 7-azatryptophan into proteins (see also refs *12* and *13*). The latter two analogues can yield what have been called "spectrally enhanced" proteins, since these analogues absorb at longer wavelengths than does Trp itself. For example, 5-hydroxytryptophan (5HW)[1] and 7-azatryptophan (7AW) both have absorbance shoulders that extend to about 320 nm. In fluorescence experiments, this enables selective excitation of the analogues, even in the presence of other absorbing tryptophans, tyrosines, or nucleic acid bases. Ross, Szabo, and Hogue (*6*) have recently published a review of methods for incorporation of a Trp analogue into a protein and the resulting fluorescence and absorbance properties of these modified proteins, which can be called "alloproteins" (*14*).

There has been less work, to date, to characterize the effect of these analogues on the structure and stability of proteins, or a set of homologous proteins. We have recently described the biosynthetic incorporation of two Trp analogues, 5HW and 7AW, into Staphylococcal nuclease A (*15*). We selected this protein, since it has been so widely studied as a standard protein for understanding the kinetics and thermodynamics

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[1] Abbreviations: 4FW, 4-fluorotryptophan; 5FW, 5-fluorotryptophan; 6FW, 6-fluorotryptophan; 5HW, 5-hydroxytryptophan; 7AW, 7-azatryptophan; CD, circular dichroism; V66W, mutant of nuclease with V66 replaced by W66; V66W′, the Δ137−149 fragment of V66W, which lacks the last 13 amino acid residues

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of unfolding and folding (*16*, *17*). Also, there are hundreds of mutants available for nuclease.

In this set of papers, we present data for the incorporation and characterization of a number of Trp analogues into three forms of nuclease: the wild-type protein, its V66W mutant, and the Δ137−149 fragment of the latter mutant. The reason for selecting these three proteins is that wild-type nuclease has a single Trp at position 140 (near the end of the long C-terminal helix of the protein), the V66W mutant has an additional Trp at position 66 (located in the β-barrel core of the protein), and the Δ137−149 fragment of V66W (to be referred to as V66W′) has only a Trp at position 66 (*16*, *18*, *19*). Thus this set of proteins enables the characterization of the Trp analogues placed at two different positions in the protein. Also, the thermodynamics of the unfolding of these three proteins has been studied (*16*, *18*, *24*). Whereas the unfolding of the wild-type protein is well-described as a two-state process, the unfolding of V66W is less cooperative and can be described as a three-state process. The fragment V66W′ is much less stable than the wild type, as is the case for all such Δ137−149 fragments of nuclease (although V66W′ is among the most stable of such fragments).

In the first of these three papers we report fluorescence and circular dichroism studies of these three proteins, prepared to contain one of several Trp analogues, including 5HW, 7AW, 4-fluorotryptophan (4FW), 5-fluorotryptophan (5FW), and 6-fluorotryptophan (6FW). The focus of this first paper is to establish the degree of analogue incorporation, to characterize any obvious structural changes upon incorporation, and to evaluate the utility of the analogues as spectral probes. In the second paper we report studies of the thermal and denaturant-induced unfolding of these proteins to quantitatively characterize the effect of the analogues on the thermodynamic stability of the alloproteins. In the third paper we report studies of the triplet state of these alloproteins.

MATERIALS AND METHODS

Materials. L-Tryptophan, L-5-hydroxytryptophan, D,L-7-azatryptophan, D,L-4-fluorotryptophan, D,L-5-fluorotryptophan, D,L-6-fluorotryptophan, guanidine hydrochloride (molecular biology grade), and fast-flow S-Sepharose (Pharmacia) were obtained from Sigma Chemical Company (St. Louis, MO). Bacto-agar (Difco), Bacto-yeast extract (Difco), Bacto-tryptone (Difco), and agarose were obtained through Fisher Scientific (Pittsburgh, PA). Biotin, MOPS, and tricine were obtained from Amresco (Solon, OH).

Methods. The expression system for WT nuclease, V66W, and V66W′ has been described elsewhere (*15*, *21*). The gene encoding each of the proteins (the gene is on a plasmid isolated from *E. coli* AR120) was transformed into Trp auxotroph UM1. The expression of the gene is under control of the λ P_L promoter and is regulated by a temperature-sensitive λ repressor. To incorporate the analogues into the target proteins, we induced the cells in the presence of the following supplements of tryptophan analogues: Trp, 20 μg/mL (induced for 2 h; 41 mg yield of nuclease/L growth); 5HW, 40 μg/mL (4 h; 40 mg of nuclease, 45 mg of V66W, 41 mg of V66W′); 7AW, 20 μg/mL (3 h; 51 mg of nuclease, 26 mg of V66W, 66 mg of V66W′); 4FW, 40 μg/mL (3 h; 70 mg of nuclease, 70 mg of V66W, 130 mg of V66W′); 5FW, 40 μg/mL (3 h; 68 mg of nuclease, 97 mg of V66W, 134 mg of V66W′); and 6FW, 20−40 μg/mL (3 h; 74 mg of nuclease, 92 mg of V66W, 108 mg of V66W′). The proteins were found to be electrophoretically pure on denaturing gels.

Molar extinction coefficients were calculated using the protein concentrations determined by the Lowry assay, using wild-type nuclease as the standard. Enzymatic activity was measured by the method of Cuatrecasas et al. (*22*).

Circular dichroism spectra were measured on an Aviv 62DS spectropolarimeter (Aviv Instruments, Lakewood, NJ). Protein samples were approximately 30 μM in 20 mM NaH_2PO_4, pH 7.3. All spectra were recorded at 20 °C, which was controlled with a thermoelectric cell holder. CD spectra (an average of triplicate runs) in the aromatic region (260−360 nm) were collected in a 1 cm path length cell, with 0.6 nm resolution (bandwidth) and 6 s integration times. The buffer baseline was subtracted, and all spectra are raw spectra (i.e., not smoothed). CD spectra (an average of triplicate runs, again not smoothed, with buffer baseline subtracted) in the far-UV region (180−260 nm) were collected using a 0.02 cm path length cell with a 3 s integration time. The far-UV spectra thus obtained were analyzed for α-helix, β-sheet, and "other" content using the algorithm of Sreerama and Woody (*23*).

Steady-state fluorescence spectra were measured on a Perkin−Elmer MPF-44A spectrofluorometer. The emission and excitation bandwidths were 5 nm, and temperature was controlled using a water-jacketed cell holder. Fluorescence quantum yields, Φ, of the protein were determined relative to the value of 0.13 for Trp using the relationship $\Phi = F_{protein}A_{Trp}/(F_{Trp}A_{protein})$, where F is the integrated fluorescence intensity and A is the absorbance of the respective sample, using the same excitation wavelength.

Time-resolved fluorescence decay data were obtained using a frequency domain instrument (ISS Inc., Urbana, IL), with an argon ion laser for excitation (selecting the lines at 300 or 305 nm using interference filters). Data were collected at 20 °C using *p*-terphenyl as a reference ($\tau = 1.0$ ns), with a modulation frequency typically ranging from 10 to 200 MHz. The modulation and phase data were fitted by a biexponential decay law, as described elsewhere (*25*).

^{19}F NMR measurements (wtihout proton decoupling) were made with a Bruker 500 DRX. Protein was dissolved to a concentration of approximately 0.3−0.5 mM in 2 M guanidine−HCl, 0.02 M sodium phosphate, pH 7.1, and 10% D_2O. The samples were also spiked with 0.5 mM D,L-5-fluorotryptophan.

RESULTS

Nuclease, V66W, and V66W′ were produced to contain each of the Trp analogues. The amount of nuclease obtained was 5−23% of the whole cell protein mass. Since we induced for different lengths of times and with different concentrations of the analogues (and we used D,L mixtures for all except Trp and 5HW), we cannot draw any conclusions about the differences in production with the different analogues. Without the addition of Trp or analogue, less than 5 mg of nuclease was isolated following induction. The various wild-type nucleases and V66W nucleases were found to have enzymatic activity that was within a factor of 2.5 of

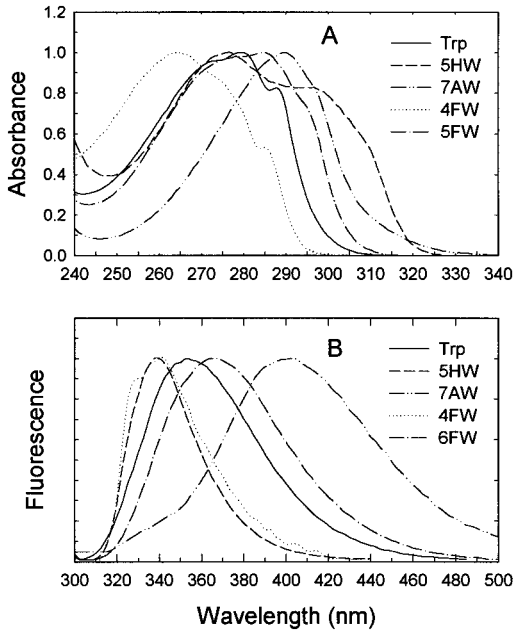

FIGURE 1: (A) Absorbance spectra of free tryptophan analogues, in 0.1 M sodium phosphate buffer at pH 7.3, room temperature. The spectrum of 6FW is not shown, since it is very similar to that of Trp. (B) Steady-state fluorescence spectra for free tryptophan analogues in pH 7.3 phosphate buffer, 20 °C. Excitation at 295 nm. Spectra are normalized to have the same maximum intensity. The fluorescence of 5FW is not shown, since it is very similar in shape and only ~7 nm redder than that of Trp.

Table 1: Spectroscopic Properties of Free Trp Analogues[a]

	absorbance		fluorescence		
analogue	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	λ_{max} (nm)	Φ	$\langle\tau\rangle$ (ns)
tryptophan	280 (288)	5400	353	0.13	2.8
5-hydroxytryptophan	277 (298)	4800	339	0.256	3.6[b]
7-azatryptophan	290	6000	403	0.016	0.75[b]
4-fluorotryptophan	265 (285)	3300	341	0.001	nd
5-fluorotryptophan	285	5400	360	0.14	2.7
6-fluorotryptophan	281	4900	366	0.14	4.2

[a] All ϵ are for 280 nm and quantum yields are with excitation at 280 nm. Values in parentheses under absorbance indicate shoulders. The $\langle\tau\rangle$ values are the mean fluorescence lifetime, $= \sum \alpha_i\tau_i$, where α_i and τ_i are the amplitude and lifetime for a biexponential fit. All fluorescence decays were multiexponential. All data are for aqueous phosphate buffer, pH 7.3 at 20 °C. [b] From ref *3*.

the Trp-containing wild-type nuclease. We did not attempt a thorough study of these enzymatic activities; our interest was only to see if the alloproteins were enzymatically active.

Absorbance and Fluorescence Spectra of the Free Analogues. Shown in Figure 1A are normalized absorbance spectra of the various free (in pH 7.3, 0.1 M phosphate buffer) Trp analogues. Most of the analogues have red-shifted absorbance spectra, as compared to Trp. This red shift is dramatic for 5HW and 7AW. Among the fluorotryptophans, 5FW also has a red-shifted absorbance. 4FW, on the other hand, has a blue-shifted absorbance. The spectrum for 6FW is very similar to that for Trp and is not shown. The molar extinction coefficients of these analogues, at 280 nm, are given in Table 1.

Shown in Figure 1B are the steady-state emission spectra of the free Trp analogues. The spectra have normalized maximum intensities in this figure. 5HW has a blue-shifted

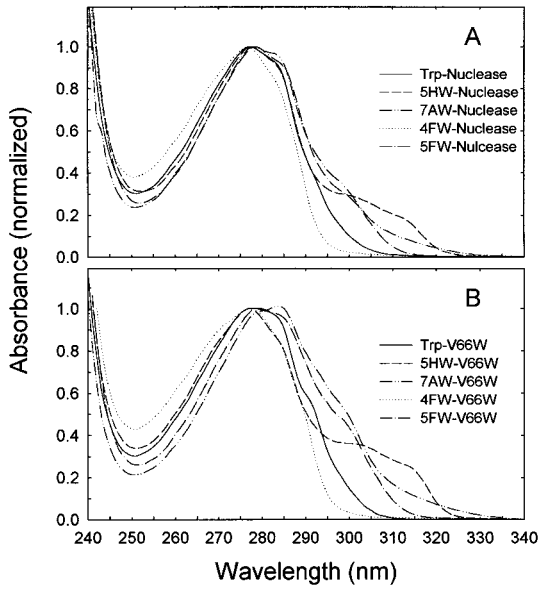

FIGURE 2: (A) Absorbance spectra of wild-type nuclease alloproteins, containing the Trp analogues at position 140. The spectrum of 6FW-nuclease is not shown, since it is very similar to that of Trp-nuclease. (B) Absorbance spectra of V66W alloproteins, containing the Trp analogues at positions 66 and 140. The spectrum of 6FW-V66W is not shown, since it is very similar to that of Trp-V66W.

and narrow emission. The emission of 7AW is red-shifted to a maximum of 403 nm. 5FW and 6FW have a slightly red-shifted emission, but 4FW has very blue emission. The emission maximum of these analogues is given in Table 1. Also given in this table are the fluorescence quantum yields of these analogues. Most notable is the extremely low quantum yield of 4FW and 7AW.

Absorbance and Fluorescence Spectra of Alloprotein Forms of Nuclease, V66W, and V66W′. The normal, Trp-containing form of WT nuclease and the fragment V66W′ each has a single Trp, at positions 140 and 66, respectively. The mutant V66W has Trp's at both positions. In considering the absorbance and fluorescence spectra of these proteins, these contents must be kept in mind.

In Figure 2A are shown the absorbance spectra of WT nuclease containing Trp, 5HW, 7AW, and two of the three fluorotryptophans. Obvious differences are observed, compared to the Trp-containing protein, indicating the successful incorporation of the analogues. The change with 6FW is small (not shown), which is expected since the absorbance of this analogue is similar to that of Trp itself. The incorporation of 4FW results in a noticeable blue shift in the absorbance spectrum. In Figure 2B are shown absorbance spectra for V66W alloproteins. Again, there are obvious spectral shifts when 5HW, 7AW, and the fluorotryptophans are incorporated, indicating the high degree of incorporation of these analogues. Since there are two Trp/analogue sites for this mutant, the spectral changes are larger than those for the WT protein.

We have previously reported the incorporation of 5HW and 7AW into WT nuclease and have shown that we can simulate the absorbance spectrum of these alloproteins in terms of contributions from the various aromatic amino acids (actually this was done for the unfolded protein in 5 M guanidine−HCl to minimize local environment effects of the absorbance spectra) (*15*). The results of fitting the absor-

Table 2: Percent Incorporation of Trp Analogues, Calculated by Various Methods

protein	analysis of absorbance spectrum	other evidence
	Wild-Type Nuclease	
5HW	0.95 5W; 0.05 Trp	
7AW	0.98 7AW; 0.02 Trp	fluorescence yield, 0.85 7AW fluorescence decay, 0.90 7AW
4FW	0.97 4FW; 0.03 Trp	NMR integration, 0.90 ± .1 4FW fluorescence yield, 0.65 4FW
5FW	1.22 5FW; −0.22 Trp	NMR integration, 0.95 ± .1 5FW
6FW	nd	NMR integration, 1.05 ± .1 6FW
	V66W Nuclease	
5HW	1.78 5HW; 0.22 Trp	
7AW	1.99 7AW; 0.01 Trp	fluorescence yield, >1.8 7AW
4FW	1.81 4FW; 0.19 Trp	fluorescence yield, >1.0 4FW
5FW	2.63 5FW; −0.63 Trp	
6FW	nd	
	V66W′ Nuclease Fragment	
5HW	0.87 5HW; 0.13 Trp	
7AW	1.05 7AW; −0.05 Trp	fluorescence yield, >0.90 7AW
4FW	0.82 4FW; 0.18 Trp	
5FW	1.32 5FW; −0.32 Trp	
6FW	nd	

bance spectra, given in Table 2, show that >95% of WT nuclease alloproteins contain the analogues 5HW or 7AW, respectively, at position 140. A similar analysis for the 5FW and 4FW analogue-containing proteins indicates that the WT proteins contain between 90% and 130% of these analogues at the tryptophan sites. Since 6FW has an absorbance spectrum that is very similar to that of Trp, the spectra fitting procedure will not distinguish this analogue from Trp itself. Fitting the absorption spectra for the 5FW-containing proteins seems to lead to an overestimate of the potential incorporation of this analogue, which indicates that the absorption spectrum of 5FW in proteins is not well modeled by free 5FW. For V66W and V66W′ alloproteins, fitting of the spectra indicates a significant degree of incorporation of the respective analogues, as listed in Table 2. For example, we estimate the percent incorporation of 5HW and 7AW into positions 66 and 140 of V66W and V66W′ to be >87%.

The steady-state fluorescence (normalized to have the same intensity) of the various alloprotein forms of WT nuclease is shown in Figure 3A. A wide range of emission maximum is observed, extending from 328 nm for 4FW-containing protein to 355 nm for 7AW-containing protein. The fluorescence quantum yields of these proteins are reported in Table 3, along with the emission maximum values.

Regarding V66W and V66W′, we have previously shown that the Trp emission of Trp-66 in the fragment is rather blue, with a maximum at 326 nm (*24*). The fluorescence of V66W is a composite of the emission of Trp-66 (maximum of ∼326 nm) and Trp-140 (maximum of ∼337 nm, as in the WT protein), thus giving an intermediate emission maximum for V66W. These results are for the Trp-

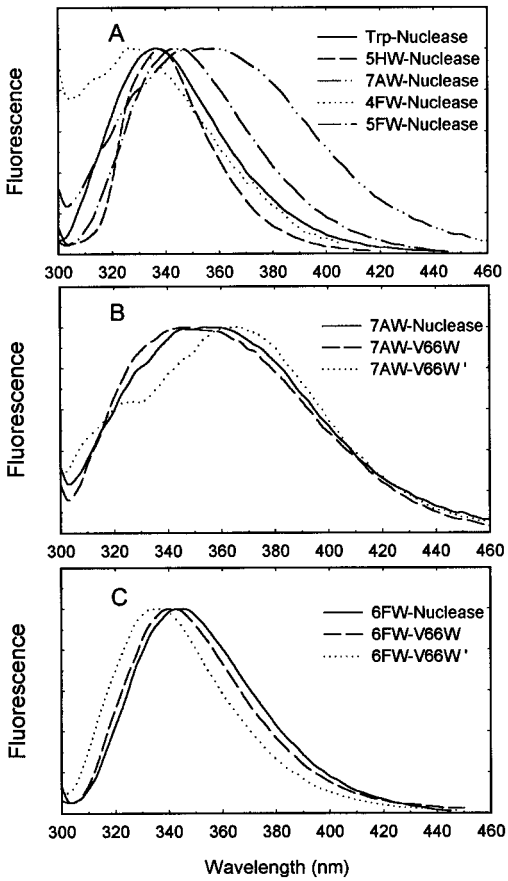

FIGURE 3: (A) Steady-state fluorescence spectra of WT nuclease alloproteins. Excitation at 295 nm, 20 °C, pH 7.3. Spectra are normalized to have the same maximum intensity. The emission of 5FW-nuclease is not shown, since it is only ∼3 nm red-shifted from that of Trp-nuclease. (B) Comparison of the fluorescence of the set of proteins, WT, V66W, and V66W′, containing 7AW. (C) Comparison of the fluorescence of the set of proteins, WT, V66W, and V66W′, containing 6FW.

containing proteins. In Figure 3B,C we show the fluorescence spectra of the 7AW- and 6FW-containing set of proteins. Table 3 summarizes these results. For the alloprotein forms of V66W′ and V66W containing the fluorotryptophans, the general pattern of λ_{max}(WT) > λ_{max}(V66W) > λ_{max}(V66W′) is the same. That is, the emission of V66W′ (position 66) is relatively blue, and the emission of V66W (both positions) seems to be a composite of the emission from both analogues and thus has a maximum intermediate between that of the respective WT and V66W′ alloproteins. Exceptions to this pattern are seen for the 5HW and 7AW series of proteins. The emission λ_{max} of 5HW is not sensitive to differences in the environments of positions 66 and 140. For the set of proteins containing 7AW, the red emssion (λ_{max} = 365 nm) for the V66W′ fragment appears to be due to the fact that this alloprotein is more unfolded than are the V66W′ variants having Trp or the other analogues at position 66. This very marginal stability of 7AW-V66W′ will be characterized in the following article.

Time-Resolved Fluorescence Data. The fluorescence lifetimes of the various analogue-containing forms of WT nuclease were determined via frequency domain measurements. The data were fitted by a biexponential decay law. The resulting mean decay times, $\sum \alpha_i \tau_i$, are given in Table 3 for all WT alloproteins (except that with 4FW, for which

Table 3: Absorbance and Fluorescence Properties of Proteins

protein	ϵ_{280} (M^{-1} cm^{-1})	λ_{max} (nm)	quantum yield[c]	$\langle\tau\rangle$ (ns)	ϕ_1 (ns) (%β)
		Wild-Type Nuclease			
Trp	16 600[b]	336	0.191	5.20	12.7
	15 900[c]				(90%)
5HW	16 000[b]	338	0.129	3.55	12.6
	15 300[c]				(95%)
7AW	15 900[b]	355	0.047	2.62	nd
	16 500[c]				
4FW	15 500[b]	328	0.03	nd	nd
	13 800[c]				
5FW	16 900[b]	339	0.13	3.87	14.3
	16 600[c]				(88%)
6FW	16 700[b]	344	0.25	8.05	12.4
	15 400[c]				(100%)
		V66W Nuclease			
Trp	23 500[b]	333	0.20	4.94	11.8
	21 300[c]				(92%)
5HW	20 400[b]	338	0.143	nd	nd
	20 200[c]				
7AW	21 900[b]	346	0.035	nd	nd
	22 400[c]				
4FW	18 800[b]	328	0.05	nd	nd
	17 200[c]				
5FW	23 000[b]	338	0.15	4.08	13.3
	22 700[c]				(95%)
6FW	23 300[b]	341	0.22	7.52	11.9
	20 200[c]				(93%)
		V66W' Nuclease Fragment			
Trp	17 800[b]	325	0.21	4.61	16.7
	15 900[c]				(62.5%)
5HW	16 100[b]	338	0.089	4.34	14.7
	15 300[c]				(92%)
7AW	18 100[b]	365	0.031	nd	nd
	16 500[c]				
4FW	15 300[b]	324	0.08	nd	nd
	13 800[c]				
5FW	17 900[b]	330	0.18	4.40	14.5
	16 600[c]				(81%)
6FW	18 300[b]	336	0.24	6.87	11.8
	15 400[c]				(86%)

[a] The quantum yields were determined with 295 nm excitation, except for 4FW, which was excited at 290 nm. All fluorescence decays are multiexponential. $\langle\tau\rangle = \sum \alpha_i\tau_i$ is the mean fluorescence lifetime for a biexponential decay fit, where α_i and τ_i are the relative fluorescence amplitude and decay times for component i. ϕ_1 is the long rotational correlation time for a biexponential decay fit of fluorescence anisotropy decay data. The short rotational correlation time, ϕ_2, was fixed at 0.3 ns in the fits; $\%\beta_1$ is the fractional preexponential term for the fit, where $\%\beta_1 = \beta_1 r_o/\sum \beta_i r_o$ and r_o is the limiting anisotropy (e.g., r_o is 0.32 for tryptophan with excitation at 300 nm). Note that the r_o for some of the tryptophan analogues is lower; for example, the r_o of 5FW is 0.15 at this wavelength. [b] Molar extinction coefficients determined by direct protein concentration determination using a Lowry assay. [c] Molar extinction coefficients calculated using the Pace et al. (*20*) equation and the extinction coefficients in Table 1 for the Trp analogues.

the absorbance is too small at 300 nm for excitation with our laser). Differential polarized phase and modulation (anisotropy decay) data were also obtained for the WT nuclease alloproteins. The data were fitted with a biexponential decay law to obtain a long rotational correlation time, ϕ_1, for global rotation of the protein and a short correlation time, ϕ_2, for segmental motion of the fluorophore side chain. The following values of ϕ_1 were obtained: 12.7 ns for Trp-nuclease; 12.6 ns for 5HW-nuclease; 14.3 ns for 5FW-nuclease; and 12.4 ns for 6FW-nuclease (7AW-nuclease and 4FW-nuclease were not studied due to their low emission intensities and/or low limiting anisotropies at 300 nm). In each case the amplitude associated with the long ϕ_1 value is >88% of the sum of the amplitudes associated with both ϕ_1 and ϕ_2, which indicates that the segmental motion of the fluorophore is limited to a cone angle of less than 16−17° (*25*, *26*).

CD Spectra. The aromatic CD spectrum of WT nuclease containing 5HW and 7AW has previously been reported (*24*). As expected from the red-shifted absorbance of these two chromophores, the aromatic CD of these respective proteins also extends to about 315 nm (see Figure 5 of Wong and Eftink (*15*)). The aromatic CD spectra of the fluorotryptophan-containing WT nuclease forms are fairly similar to that for the Trp form of the protein, with the 5FW form having a slightly redder aromatic CD spectrum and the 4FW form having a slightly bluer aromatic CD spectrum. The far-UV CD spectra of the 5HW, 7AW, and all three fluorotryptophan forms of WT nuclease are all very similar (see Figure 5 of Wong and Eftink (*15*)). Analysis of the spectra for secondary structure components using the algorithm of Sreerama and Woody (*23*) yields 38−44% α-helix, 15−18% β-sheet, and 23−28% random for the set of proteins.

The aromatic CD spectra of V66W′ and V66W (tryptophan-containing) are quite different from that for Trp-140 in WT nuclease (see Figure 3 of Eftink et al. (*24*)), since the aromatic CD region is sensitive to the different three-dimensional environment of positions 66 and 140. The differences are also seen for the various alloprotein forms of these proteins containing 5HW, 7AW, and the fluorotryptophans. Shown in Figure 4 are the aromatic CD spectra of a few selected cases, the 5HW-, 7AW-, and 6FW-containing forms of V66W′ and V66W.

The far-UV CD spectra of the same selected alloprotein forms of V66W′ and V66W are shown in Figure 5. For the full-length mutant, V66W, the far-UV CD spectrum is fairly similar for the Trp, 5HW, and various fluorotryptophan forms; in each of these cases, the spectrum is also similar to that for WT nuclease, indicating a similar degree of secondary structure for these proteins. For 7AW-containing V66W, however, the ellipticity in the 210−230 range and the band at 190 nm are notably reduced in magnitude, suggesting some loss of structure.

The Trp-containing (Trp-66) fragment V66W′ has a far-UV CD spectrum that indicates a loss of α-helix content (*24*). The same seems to apply for the forms of V66W′ having the Trp analogues, as shown in Figure 5. All the V66W′ variants have a far-UV CD spectrum similar to that for Trp-V66W′, with the exception of 5HW-V66W′, for which the minimum at 200 nm is even larger, suggesting that the latter has more random structure.

^{19}F NMR Measurements. We have performed some preliminary ^{19}F NMR measurements to confirm that the fluorotryptophans have been biosynthetically incorporated into the proteins. Using free 5FW as a reference, we have integrated the NMR signals for the proteins (in 2 M guanidine−HCl) to calculate the percent incorporation of the various fluorotryptophans. The results are shown in Table 2.

DISCUSSION

Evidence for Incorporation of the Trp Analogues. Several lines of evidence indicate that we have achieved a significant level of incorporation of 5HW, 7AW, and the three fluoro-

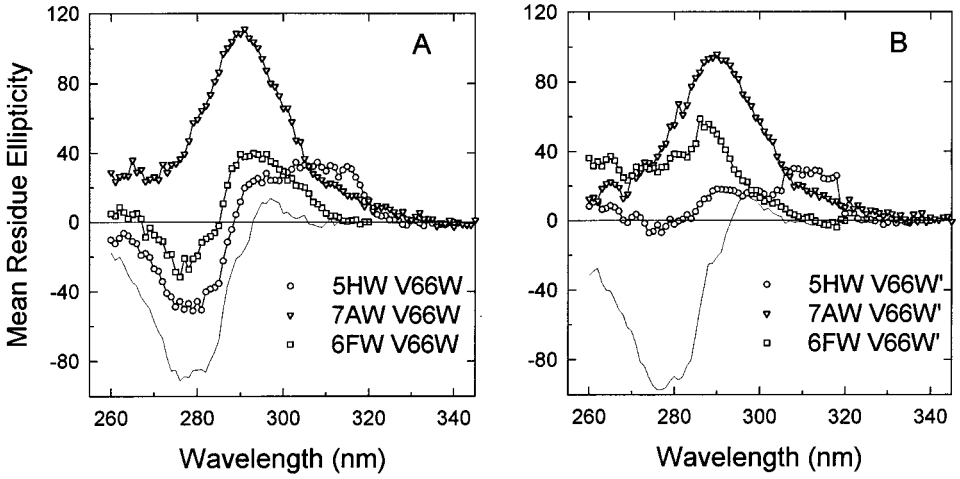

FIGURE 4: Aromatic CD spectra of V66W (A) and V66W′ (B) containing 5HW, 7AW, and 6FW. The faint solid curve is the aromatic CD spectrum of Trp-nuclease for comparison. The spectra of Trp-V66W and Trp-V66W′ are shown in Figure 3 of ref *24*.

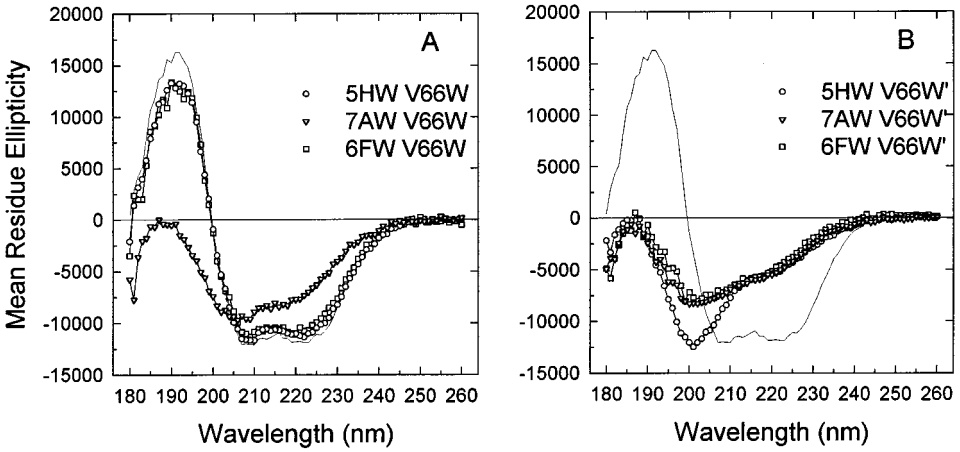

FIGURE 5: Far-UV CD spectra of V66W (A) and V66W′ (B) containing 5HW, 7AW, and 6FW. The faint solid curve is the CD spectrum of Trp-nuclease for comparison. The spectra of Trp-V66W and Trp-V66W′ are shown in Figure 3 of ref *24*.

tryptophans into WT, V66W, and V66W′ nuclease. We first summarize this evidence, since it will be important in the interpretation of certain data to have an estimate of the extent to which the protein contains the analogue or residual Trp.

(1) The amount of protein produced by the expression system without added analogue supplement is less than 5 mg, whereas between 25 and 130 mg is produced when the various analogues are added.

(2) The changes in the absorbance spectra (see Figure 2) indicate the incorporation of the analogues (though the absorbance change with 6FW is too small to be convincing). From simulation of the absorbance spectra of the guanidine−HCl denatured proteins, we calculate the percent incorporation given in Table 2.

(3) The fluorescence emission spectra of the various proteins show characteristic changes (see Figure 3), indicative of analogue incorporation. We have considered whether we can use the fluorescence spectra and the quantum yields of the free analogues to simulate the fluorescence spectra of the proteins and hence calculate the analogue content (as done with the absorbance spectra). However, this would require certain assumptions. That is, we could assume that the quantum yields and emission maximum of the analogues are unaffected by being incorporated into a protein. These are the types of assumptions made in simulating the absorbance spectra; however fluorescence signals are broad and have less features than do absorbance spectra, and fluorescence signals are generally too sensitive to the environment for this type of simulation of spectra. A rough estimation can be made, however, in the cases of 4FW- and 7AW-containing alloproteins, since the fluorescence quantum yield of these is so much lower than that of the corresponding Trp alloproteins. We make this estimation by assuming that the apparent quantum yield, Φ_{app}, of analogue-containing protein is equal to

$$\Phi_{app} \approx \Phi_{analog} X_{analog} \epsilon_{Trp}/\epsilon_{analog} + \Phi_{Trp} X_{Trp}$$

where X_{analog} and X_{Trp} are the mole fractions of the protein that contain the analogue and residual Trp, Φ_{analog} and Φ_{Trp} are the quantum yields of the free analogue and Trp, and ϵ_{analog} and ϵ_{Trp} are the extinction coefficients for the two at the excitation wavelength (295 nm; the use of lower wavelengths would further complicate matters by excitation of tyrosine residues). Using this admittedly shaky strategy, we estimate that the 4FW- and 7AW-containing WT nucleases have >65% and >85% of the analogue incorporated, respectively. Similar calculations are given in Table 2 for the other cases with 4FW and 7AW. In some cases, this type of calculation yields a lower estimate of the analogue incorporation than simulating the absorbance spectra. We place more credence in the latter, due to the above-mentioned

assumptions of analyzing the quantum yields and to the additional complication that a small amount of tyrosine excitation and emission (and energy transfer to the Trp or analogue) will occur even when exciting at 295 nm; these contributions will compromise the use of the above equation.

(4) Phosphorescence spectra, to be presented in the third paper in this series, also show characteristic spectra for the alloproteins that indicate a high degree of incorporation of the various analogues. In some cases, however, the very sharp 0−0 transition for Trp at ∼405−410 nm provides fingerprint evidence that there is some residual Trp in some of the proteins. Such phosphorescence spectra are difficult to quantitatively analyze due to the unknown efficiencies of intersystem crossing for the analogues.

(5) The aromatic CD spectra for the 5HW- and 7AW-containing proteins show signals that extend to 320 nm, which indicates contributions from these two Trp analogues.

(6) Analysis of the time-resolved fluorescence data, in a few cases, can provide some evidence for analogue incorporation. Since Trp at either position 66 or position 140 is known to be dominated by a long decay time of approximately 5 ns, we can make a rough estimate of the percent of residual Trp in an alloprotein, if the analogue has a decay profile that is distinctively different from that of Trp (as in the case of 7AW). We have fitted the decay data for the 7AW-containing WT nuclease by fixing a component to have a 5.2 ns decay time (then determining the amplitude of this component). Such an analysis for nuclease-7AW is consistent with there being as much as 20% residual Trp. (We have not made time-resolved fluorescence measurements with 4FW-containing proteins, which would also be expected to have decay times much different from the pattern for Trp, since we are limited to excitation at 300−305 nm with our laser and there is very little absorbance of the 4FW proteins in this wavelength range.)

Interpretation of CD and Fluorescence Data. Since we conclude that we have a relatively high degree of incorporation of the analogues into each protein, we can suggest some interpretations of the fluorescence and CD data. For some types of data (i.e., fluorescence with excitation at or above 305 nm, ^{19}F NMR), there is less concern about contamination by residual Trp, since the method is selective for the analogue. We will focus on interpretations of fluorescence and CD data in this paper, with triplet state studies and NMR studies to be presented elsewhere.

Regarding the fluorescence results, two major conclusions from the data reported here are that (a) the various Trp analogues show characteristic fluorescence data, and (b) the fluorescence from position 66 and position 140 is different.

5HW has an absorbance that extends to 320 nm, and thus, it can be preferentially excited, in the presence of other Trp residues or nucleic acid bases, at wavelengths of 305−320 nm (*6*). Despite this extended red excitation band, 5HW has a bluer fluorescence (i.e., a small Stokes shift), as compared to Trp, and its fluorescence spectrum is not sensitive to its environment. For example, the emssion λ_{max} of 5HW is 338−339 nm, whether free or incorporated into nuclease. Thus the primary spectral advantage of 5HW is its red absorbance. This lack of environmental sensitivity of 5HW emission appears to be due to the fact that the ^1L_b transition oscillator is S1 (i.e., the lowest energy excited singlet state, from which emission occurs) for 5HW, whereas ^1L_a is S1 for Trp in polar environments (*27*−*29*). As a result of this extended ^1L_b excitation band in 5HW, the anisotropy excitation spectrum of this fluorophore shows a fairly extended limiting plateau from 305 to 320 nm. This plateau region should be an advantage for anisotropy decay measurements, but a tradeoff (in comparison with Trp) is that the limiting anisotropy, $r_o = 0.25$, of 5HW at the red excitation edge is lower than the $r_o = 0.32$ of Trp (*27*). (See below for discussion of anisotropy decay results for Trp-nuclease and 5HW-nuclease.)

7AW has a redder emission, and its emission maximum appears to be very sensitive to the environment, as has been found by other studies (*6*, *12*, *31*−*36*). The λ_{max} of 7AW ranges from 403 nm for the free fluorophore to 346 nm in 7AW-V66W. Such a blue shift in the emission of 7AW has previously been found for the incorporation of this analogue into tryptophanyl−tRNA synthetase, either into position 92 or as bound 7AW-AMP (*3*). The fluorescence quantum yield of free 7AW is very low in neutral aqueous solution ($\Phi = 0.007$ with excitation at 295 nm; we find the yield of free 7AW to depend on excitation wavelength). Upon incorporation of 7AW into position 140 of nuclease, there is both a decrease in the Stokes shift ($\lambda_{max} = 355$ nm) and a large increase in quantum yield ($\Phi = 0.05$). Like 5HW, the limiting anisotropy ($r_o = 0.16$) value, approached at the long wavelength plateau between 310 and 325 nm, is lower for 7AW than the limiting anisotropy for Trp (*15*). Also the limiting anisotropy of 7AW is only about 0.05 at 300 nm, which means that there will be a small amplitude for anisotropy decay measurements at this wavelength.

With 5FW, both the absorbance and fluorescence spectra of each protein are slightly red-shifted, as compared to the Trp-containing forms. There is very little difference in absorbance and fluorescence of 6FW, as compared to Trp, forms of the proteins. In contrast, 4FW has very distinctive spectral properties, which potentially makes it a fluorescence "knock-out" analogue (*6*, *37*). It has a very low fluorescence quantum yield and blue emission when free in aqueous solution. When incorporated into WT nuclease, V66W, or V66W′, 4FW has a much higher quantum yield of 0.03−0.08. There is a question as to whether this fluorescence is due to 4FW or residual Trp (or is a sum of tyrosine and residual Trp emission). Although it is possible that the fluorescence of residual Trp is dominating the observed emission of the 4FW proteins, the spectral shapes show clear maxima at the indicated wavelengths (i.e., 328 nm for nuclease-4FW), not peaks at 308 or 336 nm that would be expected for tyrosine or Trp emission. If fluorescence with $\Phi \approx 0.03-0.08$ is being observed for 4FW in a protein, this means that the quantum yield of 4FW has increased 30−80-fold upon burial in a protein interior. A concern about 4FW is that it undergoes facile photodecomposition upon exposure to UV light (*30*). Even if 4FW has a low fluorescence quantum yield when incorporated into proteins, this analogue still can be very useful for forming fluorescence "knock-out" proteins. This is because the absorbance of 4FW is bluer than that of Trp, enabling excitation of Trp residues (i.e., in another protein, when studying protein−protein interactions) at 300 nm in the presence of 4FW residues.

The side chain at position 66 is surrounded by nonpolar residues (assuming that there is no change in the region of

the crystal structure of nuclease in forming V66W and V66W′). Trp at position 66 in V66W′ has a blue fluorescence ($\lambda_{max} = 326$ nm), compared to the fluorescence of Trp at position 140 in WT nuclease ($\lambda_{max} = 337$ nm), a ~10 nm difference in Stokes shift for the two environments for Trp. With several of the other Trp analogues, a similar pattern is observed; the emission of the analogue at position 66 (in V66W′) is bluer than that for the analogue at position 140 (in WT nuclease). 5HW alloproteins are an exception to this pattern, since their emission is insensitive to their microenvironment. With the 7AW proteins the pattern is also different. The emission of 7AW-V66W′ is redder than that of 7AW-WT nuclease and 7AW-V66W. The red emission from 7AW at position 66 of V66W′ seems to indicate that this fragment is much more unfolded when 7AW (vs Trp) is incorporated into position 66.

Time-resolved fluorescence measurements show that the various alloproteins have nonexponential intensity decays and that, with the exception of 7AW- (and probably 4FW-, for which we could not make a lifetime determination) containing proteins, the dominant fluorescence lifetime is similar to that with Trp. The shorter mean fluorescence lifetime of 7AW is a result of its sensitivity to the environment and to various excited-state quenching reactions. Previous studies in which 7AW has been incorporated into other proteins have yielded fluorescence lifetime components ranging from 10 ns to subnanoseconds (*3*); a large range of lifetimes has also been found for 7-azaindole in different solvents (e.g., $\langle\tau\rangle$ = ~0.8 ns in water and ~6 ns in acetonitrile) (*31*, *32*). Thus, the decay time of 7AW appears to be very sensitive to the environment, and the value found for 7AW in WT nuclease is consistent with position 140 being partially removed from the aqueous phase.

Anisotropy decay data for WT nuclease alloproteins indicate that the dominant, long rotational correlation time, ϕ_1, is approximately the same in all cases. This common value of $\phi_1 \approx 13$ ns both validates the use of the Trp analogues and indicates that each of the alloproteins has a similar hydrodynamic rotational radius, which suggests that the proteins have a similar three-dimensional structure. The relative amplitude, $\%\beta_1$, of the long rotational correlation time is >90% for all WT and V66W alloproteins, indicating that there is only a small degree of segmental motion of the fluorophores. For V66W′ alloproteins, the value of ϕ_1 tends to be slightly larger (than for the WT variants) and the $\%\beta_1$ is less than 90% in most cases. This is consistent with the fragment proteins being slightly expanded and having a greater degree of segmental motion for the fluorophore at position 66.

Interpretation of CD Data. The far-UV CD spectra are similar for all of the WT nuclease alloproteins, which suggests that the secondary (and hence tertiary) structure of WT nuclease is not altered by incorporation of the analogues. The same is generally the case for V66W as well, with the exception of 7AW-containing V66W, which appears to have diminished secondary structure. These results, together with the retention of enzymatic activity and the above-mentioned similarity of the rotational correlation times, are consistent with a minimal perturbation (again, with the exception of 7AW) of the structure of wild-type nuclease and V66W by incorporation of the analogues into position 140 or 66.

The fragment, V66W′, is intrinsically less stable and partially unfolded (*18*, *24*). Consequently, one would expect the fragment to be more easily perturbed by incorporation of an amino acid analogue at position 66. The far-UV CD spectra are not clear in this matter. The Trp-containing form of V66W′ has a far-UV CD spectrum that indicates loss of most of the α-helix content present in the full-length protein. The remaining, weak CD spectrum of V66W′ is difficult to characterize, since the β-sheet spectrum is not easily distinguishable from that for a random coil. Consequently, the incorporation of 7AW, and the other Trp analogues, into position 66 of V66W′ does not result in a significant change in the appearance of the far-UV CD spectra.

SUMMARY

We have biosynthetically incorporated five different Trp analogues into WT nuclease (position 140), V66W (positions 66 and 140), and the fragment V66W′ (position 66). A high degree of incorporation is found in each case.

The various Trp analogues provide a rich set of spectroscopic probes. 5HW has a red-shifted absorbance, and its fluorescence appears to be fairly insensitive to the environment. 7AW is also red-shifted, and its fluorescence appears to be very sensitive to the environment. The fluorotryptophans each provide ^{19}F NMR probes, which have a great chemical shift dispersion. 5FW and 6FW have slightly red-shifted absorbance and have fluorescence properties that are similar to those of Trp. 4FW has a blue absorbance and fluorescence and a low quantum yield, making it nearly invisible in fluorescence measurements.

Far-UV CD, time-resolved anisotropy decay, and enzymatic activity measurements all suggest a minimal perturbation of the structure of WT and V66W nuclease by the analogues. The only exception to this statement seems to be 7AW, which reduces the secondary structure of V66W. In the following paper we will describe the effect of these substitutions on the thermodynamic stability of these alloproteins.

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